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Transcriptome assembly and profiling of *Candida auris* reveals novel insights into biofilm mediated resistance

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Running Head: RNA-Seq analysis of *Candida auris* biofilms

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ABSTRACT

Candida auris has emerged as a significant global nosocomial pathogen. This is primarily due to its antifungal resistance profile, but also its capacity to form adherent biofilm communities on a range of clinically important substrates. While we have a comprehensive understanding how other *Candida* species resist and respond to antifungal challenge within the sessile phenotype, our current understanding of *C. auris* biofilm mediated resistance is lacking. In this study we are the first to perform transcriptomic analysis of temporally developing *C. auris* biofilms, which were shown to exhibit phase and antifungal class dependent resistance profiles. A de novo transcriptome assembly was performed, where sequenced sample reads were assembled into a ~11.5 Mb transcriptome consisting of 5848 genes. Differential expression (DE) analysis demonstrated that 791 and 464 genes were up-regulated in biofilm formation and planktonic cells respectively, with a minimum 2-fold change. Adhesin related GPI-anchored cell wall genes were up-regulated at all time-points of biofilm formation. As the biofilm developed into intermediate and mature stages, a number of genes encoding efflux pumps were up-regulated, including ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters. When we assessed efflux pump activity biochemically, biofilms efflux was greater than planktonic cells at 12 and 24 h. When these were inhibited, fluconazole sensitivity was enhanced 4 to 16-fold. This study demonstrates the importance of efflux mediated resistance within complex *C. auris* communities and may explain its resilience to a range of antimicrobial agents within the hospital environment.

IMPORTANCE

Fungal infections represent an important cause of human morbidity and mortality, particularly if they adhere and grow on both biological and inanimate surfaces as communities of cells (biofilms). Recently, a previously unrecognized yeast *Candida auris* has emerged globally that has led to widespread concern due to our difficulty in treating it with existing antifungal agents. Alarmingly, it is also able to grow as a biofilm that is highly resistant to antifungal agents, yet we are unclear how it does this. Here we used a molecular approach to investigate the genes that are important in causing the cells to be resistant within the biofilm. The work provides significant insights into the importance of efflux pumps, which actively pump out toxic antifungal drugs, and therefore enhance their survival within a variety of harsh environments.

INTRODUCTION

Fungal infections affect in excess of a billion people, resulting in approximately 11.5 million life-threatening infections and more than 1.5 million deaths annually (1). *Candida auris* is an emerging fungal pathogen that has attracted considerable attention because of its ability to cause infections that are both difficult to diagnose and to treat (2). It has been responsible for a number of nosocomial outbreaks worldwide through its ability to persistently colonise and transmit between patients and the environment (3-6). Despite the unprecedented global emergence of this organism, relatively little is known about the molecular basis of its pathogenicity and antifungal resistance phenotype. The resistance profile is well documented, with >90% of isolates intrinsically resistant to fluconazole. Resistance to other azoles, polyenes and echinocandins has also been reported (4). Alarmingly, 41% of isolates have been shown to be multidrug resistant, with 4% demonstrating pan-drug resistance (4). Hot-spot mutations in *ERG11* and *FKS1* have been identified as resistance mechanisms in azole and echinocandin resistant strains respectively (7, 8).

Candida biofilms represent an important clinical entity associated with adaptive resistance to many antifungals, and are linked to excess morbidity and mortality (9-11). Although, *C. albicans* is regarded as the primary biofilm-forming pathogen within the genus, there is increasing interest and evidence for non-*Candida albicans* species biofilms (12, 13), particularly in *C. auris*. Clinically, *C. auris* has been isolated from a number of sites including wounds, line-tips and catheters, suggestive of the organism existing within a biofilm-lifestyle in host (14, 15). We recently described the ability of *C. auris* to form anti-fungal resistant biofilms, against all 3 main classes of antifungals (16), yet the mechanisms underlying this phenotype remain unknown. The speed of

discovery in this emerging pathogen has certainly been hindered by the lack of robust sequence information. Initial sequencing efforts provided a draft *C. auris* genome, however, these reads were poorly aligned to other *Candida* spp. and inconsistently annotated (17). More recently, complete and functionally annotated genome assemblies have been created, allowing the analysis of the functional capacity of the genome to be studied under clinically relevant conditions (18). Biofilm-associated resistance is a complex and multifaceted phenomenon that has been described in number of fungal pathogens. Various resistance mechanisms exist, predominately associated with the extracellular matrix (ECM), overexpression of drug targets and efflux pumps (19). Given the lack of understanding of biofilm formation and resistance mechanisms in *C. auris*, we therefore aimed to investigate these mechanisms using a transcriptomics approach.

MATERIALS AND METHODS

Microbial growth and standardisation

Four *C. auris* clinical isolates were used throughout this study (NCPF8971, NCPF8973, NCPF8984, NCPF8990) (20). Isolates were stored in Microbank vials at -80°C prior to use, before they were sub-cultured onto Sabouraud dextrose agar (SAB [Sigma, Dorset, UK]) and incubated at 30°C for 48 h. Isolates were propagated overnight in yeast peptone dextrose (YPD) media (Sigma, Dorset, UK), before washing with centrifugation as previously described (21). Cells were then standardised to 1 x 10^6 cells/mL in RPMI-1640 media and biofilms were grown in micro-titre plates, 75cm^2 tissue culture flasks, or Thermanox™ coverslips) for 4, 12 and 24 h at 37°C.

Characterisation of biofilm formation.

Isolates were standardised as described above and grown for 4, 12 and 24 h at 37°C. Following growth, biofilms were washed with phosphate buffered saline (PBS [Sigma, Dorset, UK]) and biomass quantified using the crystal violet assay, as previously described (21). In addition, biofilm composition was analysed using a propidium monoazide (PMA) qPCR, a method able to differentiate live cells from a population (22). Samples were prepared as previously described (22), before sonication in 1mL of PBS at 35kHz for 10 min in an ultrasonic water bath to remove and disaggregate the biofilm (23). After sonication, samples were incubated in the dark with 50 μM PMA (Cambridge Bioscience, Cambridge, UK) for 10 min to allow uptake of the dye. All samples were then exposed for 5 min to a 650W halogen light before DNA was extracted using the QIAamp DNA mini kit, as per manufacturer's protocol (Qiagen, Crawley, UK). One microliter of extracted DNA was then added to a mastermix

containing Fast SYBR® Green Master Mix, RNase free water and 10µM of *C auris* specific forward and reverse primers (Forward – CGCACATTGCGCCTTGGGGTA; Reverse - GTAGTCCTACCTGATTTGAGGC GAC) (24). Real-time qPCR was then used to enumerate the total of number of live cells from within the biofilm, using the following thermal profile: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Colony forming equivalents (CFE) were then calculated based upon a standard curve of serially extracted DNA ranging from 1×10^8 - 1×10^4 cells/mL.

Biofilm visualisation

Biofilms were standardised and grown on Thermanox™ coverslips (Fisher Scientific, Loughborough, UK) as described above. At selected time points, biofilms were washed with PBS before processing for scanning electron microscopy (SEM). Biofilms were fixed in 2% para-formaldehyde, 2% glutaraldehyde, 0.15M sodium cacodylate, and 0.15% w/v alcian blue, before processing as previously described (21). Biofilms were then sputter coated in gold before being viewed under a JOEL-JSM-6400 microscope.

Planktonic and sessile susceptibility testing

Planktonic minimum inhibitory concentrations (pMIC) were determined visually using the Clinical Laboratory Standards Institute M27-A3 broth microdilution method (25). Standardised cells were treated with serial two-fold dilutions of miconazole nitrate (0.25-128mg/L), micafungin (0.25-128mg/L) and amphotericin B (0.063-32mg/L). In addition, biofilms were grown for 4, 12 and 24 hr as described above before treatment with the same concentrations as planktonic cells. Sessile MICs (sMIC) were determined using the XTT metabolic reduction assay (26). The sMIC was calculated as the

concentration leading to 80% reduction in XTT-colorimetric readings in comparison to an untreated positive control.

RNA extraction and sequencing analysis

Following biofilm characterisation, *C. auris* NCPF 8973, originally isolated from a wound swab (14) was chosen for subsequent transcriptomic analysis. Biofilms were grown as described above in 75cm^3 tissue culture flasks, before being washed with PBS and biomass was dislodged using a cell-scraper. The resultant biofilm biomass was then homogenised using a bead-beater and RNA extracted using the Trizol™ (Life Technologies, Paisley, UK) method (27). Following extraction, RNA was DNase treated and purified using the RNeasy MiniElute clean up kit as per the manufacturer's instructions. Quality and quantity were assessed using a bioanalyser (Agilent, USA), where a minimum quantity of 2.5µg and a minimum quality RIN value of 7.0 was obtained for each sample. Samples were then submitted to Edinburgh Genomics (http://genomics.ed.ac.uk/) before sequencing using the HiSeq 2500 Illumina sequencer. Biological triplicates were analysed for all variables, with the exception of 4 h biofilms to which two replicates were used due to sequencing failure.

Transcriptome annotation and differential expression analysis

Raw fastq reads were quality controlled using Trim Galore v0.4.5 (https://github.com/FelixKrueger/TrimGalore) to remove Illumina adapters and trim reads with a quality score lower than 20. Reads were then aligned to the refseq genome sequence B8441 using HISAT2 (28). The aligned reads were then coordinate sorted and SAM files were converted to BAM before all aligned reads were merged using samtools (29). The resulting aligned reads were assembled *de novo* using genome

guided Trinity v2.5.1 (28). The completed transcriptome was assessed by using the contig length distribution metrics (N50), percentage of annotation and the third-party Benchmarking Universal Single-Copy Orthologs (BUSCO) v3 assessment program (http://busco.ezlab.org/). Annotation of candidate open reading frames (ORFS), identified with TransDecoder v5.0.2 (http://transdecoder.sourceforge.net/), was then performed using the Trinotate v3.1.0 package (https://trinotate.github.io/). Trinotate performs functional annotation of transcriptomes from the uniprot Swiss-Prot database via homology searches with the Basic Local Alignment Search Tool (BLAST) functions BLASTp for protein queries and BLASTx for nucleotide queries. Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG), EggNOG identifiers were also inferred from the Swiss-Prot protein database. BLAST2GO annotation was additionally performed which also relies upon BLAST but includes the annotation from European Bioinformatics Institute (EBI) InterPro databases. The extraction through to the annotation is summarised in Figure 2. The reference transcriptome created by Trinity was used to create an index the trimmed reads were then counted and annotated against this index using gene abundance quantification software Kallisto. Gene abundance files for each sample replicate were than imported into R for differential analysis based upon the DESeq2 package. All additional statistics, analysis and visualisation were produced within R. Raw data files are deposited at the following site: https://www.ncbi.nlm.nih.gov/bioproject/423159.

Temporal efflux pump activity and inhibition

The efflux pump activity of planktonic and sessile cells was assessed using the alanine β-naphthylamine (Ala-nap) fluorescent assay was used as previously described (29). For planktonic assessment, four *C auris* isolates were standardised to 5 x 10^7 cells/mL

in the assay buffer solution ($MgSO_2$ [1mM], K_2HPO_4 [50mM], and 0.4% glucose; pH 7.0). For sessile cells, biofilms were grown in black flat-bottomed microtitre plates for 12 and 24 h. Following biofilm development, these were washed with the assay buffer solution. The reaction was then initiated with the addition of 100μg/mL Ala-nap and developed for 60 min at 37°C. Fluorescence readings were obtained every 30sec using a fluorescence plate reader at an emission/excitation wavelength of 355/460nm. In addition, the efflux pump inhibitor ([EPI] L-Phe-L-Arg-β-naphthylamine dihydrochloride) was used in combination with fluconazole to determine if antifungal activity could be enhanced. Biofilms were developed in the presence of fluconazole (128-0.25 mg/L) with and without the presence of EPI at a concentration of 64 mg/L and incubated for 12 and 24 h at 37°C. Biofilms were then washed with PBS, before viability was calculated using the XTT assay as described above.

Statistical analysis

Graph production, data distribution and statistical analysis were created using GraphPad Prism (version 8; La Jolla, CA, USA) and R studio (version 1.1). For efflux pump activity experiments, data was normalised before student t test were used to compare samples. Statistical significance was achieved if $p<0.05$.

Data availability

Raw HiSeq sequencing reads (50bp) and assembly are available under sequence read archive submission: SUB3362956. Average of all gene expression values are available in supplementary datasheet 2. Gene and functional annotations are submitted in datasheet 1 and figures S1-3.

RESULTS

Candida auris biofilms exhibit temporal antifungal resistance

Mature *Candida auris* biofilms have been shown to be resistant to antifungals that are readily active against their planktonic equivalents (16). We therefore investigated the temporal effect of biofilm formation against the susceptibility to of all three major classes of antifungals. As demonstrated in Fig 1A, the maturation of *C. auris* biofilms was shown to correlate with decreased susceptibility to each antifungal agent. When assessed planktonically, the median MIC for the four isolates for miconazole was 1μg/mL, micafungin <0.25μg/mL and amphotericin B 0.5μg/mL (range 0.125-0.5μg/mL). After 4 h of biofilm development, no increases in resistance were observed against micafungin (MIC <0.25μg/mL), however the median MIC increased 16-fold to 16μg/mL (range 16-32μg/mL) for miconazole and 4-fold to 2μg/mL for amphotericin B (range 1-4μg/mL). As the biofilm matured to 12 h of growth, 2-fold increase in median MIC were shown for miconazole (range 16-64μg/mL) and amphotericin B (range 2-4μg/mL). Interestingly, the MIC was shown to significantly increase for micafungin (range 1->128μg/mL) after 12 h. After 24 h, no further increase in MIC was observed for amphotericin B. However, both miconazole and micafungin MICs were increased 2-fold to 64μg/mL and >128μg/mL, respectively.

Candida auris transcriptome assembly

Given the temporal patterns of biofilm-associated resistance, we undertook a transcriptional profiling approach to understand the mechanisms governing antifungal biofilm resistance. Sequencing of samples using Illumina HiSeq produced around 414 million single end reads of 50 bp length. Following processing, the number of reads were reduced by three million through trimming and quality control stages. All

sequenced sample reads were then assembled into a ~11.5 Mb transcriptome which consisted of 5889 identified Trinity transcripts and 5848 genes based on the longest isoform of transcripts. At least half of the assembled sequenced bases were found on contigs of a length of 3488 bp (N50) (Table 1). The completeness and quality of the *C. auris* transcriptome was assessed with BUSCO against Ascomycota (94%), Saccharomyceta (91.4%) and Saccharomycetales (91.7%) gene sets. Very small percentages of duplicate, fragmented and missing genes were observed in each of the gene sets (Table 2).

Identification by sequence homology searches with BLASTx function yielded annotation of 54% of Trinity transcripts and 54% of unique 'genes'. Identification of protein sequences with BLASTp, against Transdecoder identified ORFs and potential coding sequences, gave functional annotation matches with 51% of the transcripts and 41% of unique 'genes' (Table 1). The presence of known signal peptides, functional protein domains and protein topology was discerned by searches against the SignalP and TmHMM databases, respectively. Of the predicted proteins, 202 sequences were predicted to have signal peptides and 701 transmembrane protein topologies were predicted.

Additional annotation via the software BLAST2GO was performed, which obtains BLAST hits that are used to retrieve, and map GO and KEGG terms. It also utilises InterProScan which acquires functional annotation of protein sequences from EBI's InterPro databases (https://www.ebi.ac.uk/interpro/). These databases are consortium of online databases that include PANTHER, Pfam and SUPERFAMILY (30). Both the Trinotate and BLAST2GO annotation files are supplied as supplementary datasheet 1.

BLAST2GO searches were performed with a fungi taxonomical filter, which annotated 1157 genes with BLAST and an additional 4365 from the InterPro databases. InterPro and BLAST derived GO terms were merged to give a total of 9504 GO annotations assigned to 2,479 genes. These annotations were distributed between three main GO categories; Biological Process (3,633, 38%), Cellular Component (3116, 33%) and Molecular Function (2755, 29%) having the least (fig S1). InterProScan was able to classify Trinity transcripts according to SuperFamilys based on known structures. The most represented superfamilies were the P-loop containing nucleoside triphosphate hydrolase (236) the major facilitator superfamily (113), Armadillo-type fold (102) and Protein Kinase-like superfamily (90) (fig S2). From annotation against the available databases, there were 6 major enzyme classes represented that included: Hydrolyases (290), Transferases (150), oxidoreductases (88), ligases (42), lyases (28) and isomerases (15) (fig S3).

Differential expression and functional annotation of *C. auris* biofilms

Differential expression (DE) analysis was performed to investigate the transcriptional changes observed with biofilm development. Multivariate analysis by principal component analysis (PCA) demonstrates variance between the different time points, 0 h shows the greatest variance with the other biofilm time points. In addition, there is also some variance between biofilms at 4, 12 and 24h (Figure 3A). DE analysis demonstrated that 791 and 464 genes were up-regulated in biofilm formation and planktonic cells respectively, with a minimum 2-fold change (Figure 3A). Phase-dependent differential expression of these up-regulated genes are illustrated in the venn diagram in Figure 3B, with the down-regulated genes shown in Figure 3C, individual

genes are described in supplementary datasheet 2. Of these biofilm up-regulated genes, selected genes involved in antifungal resistance and biofilm-associated mechanisms are listed in Table 3. GPI-anchored cell wall genes including *IFF4*, *CSA1*, *PGA26* and *PGA52* were up-regulated at all time-points of biofilm formation, highlighting their potential role within cellular adhesion (Table 3). Two further adhesins, *HYR3* and *ALS5* were also shown to be up-regulated, but only in mature biofilms (Table 3). As the biofilm developed into intermediate and mature stages, a number of genes encoding efflux pumps were up-regulated including *RDC3*, *SNQ2*, *CDR1*, *YHD3*. In addition, *MDR1* was shown to be up-regulated at the 24 h time point (Table 3). To understand the functional processes related to differentially expressed genes, a cut-off of 2-fold up-regulation (adjusted p value <0.05) was used for gene ontology (G0) analysis comparing planktonic cells to 24 h biofilms. The 278 differentially expressed genes were assigned to 28 GO terms with an over enrichment *P* value <0.05, comprising 13 biological processes, 9 cellular components and 6 molecular function, and contained a number of differentially expressed functional categories (Figure 4A). Included within these GO terms were transmembrane transport, within which several ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters were highly up-regulated in *C. auris* biofilms (Figure 4B).

Efflux pumps play a primary role in antifungal resistance in *C. auris* biofilms

Transcriptional analysis and function annotation revealed a significant up-regulation of a number of drug efflux pumps, from both ABC and MFS transporters. To confirm the role of these membrane proteins within biofilms, we assessed efflux pump activity. Both 12 and 24 h biofilms exhibited increased efflux compared to planktonic cells, with 4 h biofilms below the detectable limit of the assay. Efflux from 12 h biofilms was 2.21-

fold ($p<0.05$) greater than planktonic cells, with a 2.38-fold increase shown in 24 h biofilms ($p<0.005$). No statistical differences were observed between 12 and 24 h biofilms (Figure 5). Interestingly, efflux pump activity is shown to be constitutively expressed within biofilms, with no induction observed in response to azole antifungals (Fig S4).

Given the increased activity of efflux pumps in biofilms, we then assessed the contribution of these transporters to fluconazole sensitivity (Table 4). When biofilms were incubated for 12 h in the presence of fluconazole, the $SMIC_{50}$ ranged between 32->128μg/mL. However, when also grown in the presence of fluconazole and an EPI, the $SMIC_{50}$ ranged between 2-16μg/mL for all isolates, ranging from a 4 to 16-fold increase in susceptibility. The same trend was observed for 24 h biofilms, with the $SMIC_{50}$ range between 64->128μg/mL for fluconazole only treatment, with 2-8-fold reductions observed with co-incubation with the EPI ($SMIC_{50}$ 8-64μg/mL).

Discussion

The rapid and simultaneous emergence of the pathogenic yeast *C. auris*, combined with its reported recalcitrance to all three major classes of antifungals, has led to a concerted response by the medical mycology community to understand and define the mechanisms underpinning its pathogenicity and resistance. Although preliminary investigations have investigated genetic point mutations promoting resistance (7, 8), as well as number of efflux pumps identified within its genome (17, 18), there are still substantial gaps remaining in our understanding. Moreover, irrespective of these defined chromosomally derived resistance characteristics, adaptive resistance mechanisms associated with environmental stressors is likely to be a key contributor to its success as a pathogen both in the host and environment. We have recently reported how *C. auris* exhibit enhanced pathogenicity and resistance, both *in vitro* and *in vivo*, and that the biofilm phenotype is instrumental in its lifestyle (14, 16, 31, 32). Moreover, its ability to survive and persist in the nosocomial environment, increasing the probability to cause outbreaks. We have recently reported that adherent *C. auris* cells display substrate dependent susceptibility to clinically relevant concentrations of hospital disinfectants (32), and that these biofilms were shown to be resistant to chlorhexidine and hydrogen peroxide, displaying a less susceptible phenotype than *C. albicans* and *C. glabrata* (31). Here we report for the first time that efflux-based resistance mechanisms play an important role in biofilm mediated resistance in *C. auris*, and that conserved biofilm-related genes are temporally observed, as illustrated in Figure 6.

To investigate this, we undertook an RNA-sequencing based approach for the analysis *C. auris* biofilm development, as well as profiling genes associated with resistance and

virulence mechanisms. Assembly of the transcriptome using trinity software has allowed us to construct a specific reference for our samples of interest. Additionally, annotation via numerous methods has allowed for a in depth functional characterisation of the organism. Annotation of homologs, predicted protein domains, as well as gene ontological classifications further enhances our ability to interpret mechanisms that differentiate *C. auris* under different conditions. This annotated transcriptome has been highly instrumental in expression analysis and elucidation of virulence mechanisms of *C. auris* in this and forthcoming studies.

The initiation of biofilm formation depends on an initial adherence phase of colonisation to a specific surface before subsequent proliferation to promote disease. A number of GPI-linked cell wall proteins were up-regulated at the early biofilm time-point, highlighting their role in the initial adherence stage. In *C. albicans*, IFF4 and CSA1 have been shown to be involved in adherence to both mucosal and abiotic substrates, as well as cell-cell cohesion (33-35). Transcriptional studies from Fox and coworkers (2015) identified *IFF4* as a member of a group 10 adhesion genes that are induced at the later stages of biofilm formation, and hypothesise its role mediating cell-cell contact (36). Interestingly, an iff4Δ null mutant displayed decreased adhesion at an early stage of biofilm formation, as well as attenuated virulence (37). Both studies collectively highlight its function throughout biofilm formation.

In *C. albicans*, members of the agglutinin-like sequence (ALS) proteins play a key role in the adherence of the organism, predominantly through *ALS3* (38, 39). A recent study identified that members of this cell wall protein family detected in *C. albicans* are not found in *C. auris* (18). Our analysis revealed that orthologs of only two members, *ALS1*

and *ALS5*, were represented within the *C. auris* transcriptome, with the latter up-regulated within mature biofilms. Further examination of cell wall protein families by Muñoz and coworkers (2018) failed to reveal any highly expanded families (18). It is therefore likely that a less reliant Als-dependent adherence mechanism exists for *C. auris*. Moreover, the gene encoding candidapepsin-5, commonly known as *SAP5* in *C. albicans* was shown to be up-regulated in mature biofilms. This protease is predominantly associated with its role in invasive infection (40). Indeed, studies have identified its increased expression in biofilm associated infections (41), with sap5Δ/Δ strains demonstrating a less adherent phenotype, therefore highlighting its potential as a promising biofilm biomarker (42).

One of the most defining characteristic of biofilms is their recalcitrance to antimicrobial agents. As described in other *Candida spp.* biofilm associated drug resistance comprises a number of different mechanisms that co-ordinate with one another through the various phases of biofilm development (43). An underlying mechanism across *Candida spp.* is the up-regulation of efflux pumps within biofilm associated cells (44-46). Planktonically, *C. auris* isolates displayed up to 15 times fold more ABC-transporter activity than *C. glabrata* isolates (15), highlighting a potential intrinsic azole resistance mechanism. Ramage and co-workers (2002) demonstrated that expression of *CDR1* and *MDR1* was increased within mature *C. albicans* biofilms compared to their planktonically grown equivalents, yet deletion of these genes had no effect on the susceptibility of mature biofilms (47). Indeed, temporal efflux pump analysis revealed that efflux pump mutants were more susceptible to fluconazole treatment than their parental strain at early phases of biofilm development (46), as also shown in other fungal pathogens, such as *Aspergillus fumigatus* (29). Our own temporal

analysis of *C. auris* biofilms revealed that efflux pumps were up-regulated at intermediate and mature phases of development unlike other species, though did not appear to be inducible following azole exposure. This is in contrast to analysis of *C. glabrata* biofilms exposed to azole treatment, where up-regulation of genes encoding ABC-transporters was observed (45). Muñoz *et al* (2018) recently analysed the transcriptional profile of planktonic *C. auris* in response to azole and polyene antifungals (18). After exposure of a resistant *C. auris* strain to amphotericin B almost 40 genes were shown to be differentially expressed. These included genes involved in iron transport that have previously been described in *C. albicans* to be involved in its response to amphotericin B (48). Three genes of these genes (*SIT1, PGA7, RBT5*) were shown to overlap within our own biofilm data set indicating that these may play an additional role in our observed polyene resistance.

A further key mechanism of *Candida* biofilm resistance is the formation of the ECM, which functions to provide stability and sequestration of drugs from the biofilm, as well as protection from environmental stressors (49). Recent studies have now identified that varying *Candida spp.* conserve a constitutive polysaccharide backbone that functions to impede antifungal delivery, yet the composition of the ECM varies between species (50, 51). Although its composition remains unknown, it could be hypothesised that *C. auris* ECM would be similar to that of *C. glabrata*, given the yeast cell biofilm phenotype. Temporal analysis has shown the formation of the ECM is time-dependent and associated with intermediate and maturation phases of biofilm formation (52). Our data suggests that this is similar in *C. auris*, with increased expression of *KRE6* and *EXG*, a glucan 1,3-beta-glucosidase and a close ortholog of *XOG1* in *C. albicans*, two genes involved in matrix formation (53, 54).

Given the alarming global emergence of antifungal resistance, then the requirement for new antifungals is pivotal (55). Drug efficacy and development has plateaued in recent years, yet an encouraging number of molecules remain within the antifungal pipeline (56, 57). Several studies have assessed the positive efficacy of novel compounds, including APX001, CD101, SCY078 and ceragenins against *C. auris* (58-61), which may widen the spectrum of active agents against emerging resistant species. These actives are both expansions of current drug targets, such as 1,3-β-glucan synthase inhibitors (CD101 and SCY078), as well as novel targets GPI-protein inhibitors (APX001). All of these compounds demonstrated significant *in vitro* activity against planktonic forms of *C. auris,* with APX001 also demonstrating enhanced *in vivo* efficacy compared to anidulafungin (60, 62). Although these preliminary data are very promising, there are limited studies evaluating their effect against sessile *C. auris*. The 1,3-β-glucan synthase inhibitor SCY078 was shown to significantly reduce biofilm thickness and metabolic activity after a prolonged 48 h exposure (63). Furthermore, the ceragenins, a class of antimicrobial peptide, CSA-44 and CSA-131 also demonstrated anti-biofilm activity, although the concentrations needed were 4-64 fold greater than the planktonically active equivalent (61). APX001 is a first in class compound that acts by blocking GPI synthesis through inhibition of the GPI-anchored cell wall transfer protein 1 (Gwt1). Although no such studies have been performed, then perhaps APX001 is the most attractive anti-biofilm target, given our identified function of GPI-anchored proteins in *C. auris* biofilm formation.

Given that we can now genetically manipulate this pathogenic yeast (64, 65), future work analysing the functional roles and processes of specific genes and protein will

further enhance our understanding of biofilm-associated pathogenicity and resistance. Unravelling the key factors that regulate the transcriptional network that exists for *C. auris*, similar to those studies in *C. albicans* and *C. parapsilosis* (36, 66), may provide translational insights into novel avenues for therapeutic targets for biofilm-associated infections. We have shown that efflux pumps are important during biofilm development, and this may explain why this seemingly innocuous yeast is able to survive, persist and cause continued problems within the hospital setting.

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Figure 1: *Candida auris* biofilm development correlates with increased antifungal tolerance

Candida auris biofilms were standardised $1x10^6$ CFU/mL and grown for 4, 12 and 24 h. Biofilm biomass was then quantified using the crystal violet assay, with the composition of biofilm cells enumerated using qPCR and represented by a box and whisker plot as the total biomass of four *C. auris* isolates ([left hand y-axis], A). Planktonic susceptibility testing was performed against serially diluted miconazole, micafungin and amphotericin B concentrations using the CLSI guidelines, with biofilm susceptibility testing performed using the XTT assay, with median MIC values plotted ([right hand y-axis], A). In addition, biofilms were grown, fixed and processed for SEM before imaging using a JEOL-JSM 6400 scanning electron microscope. Micrographs represent lower magnification (x1000) and higher magnification (inset, x5000) (B).

Figure 2: Bioinformatic pipeline for *Candida auris* transcriptome assembly, annotation and analysis.

Figure 3: Quality control and differential expression analysis of *C. auris* biofilms

Principal component analysis displays the largest variance along PC1 (56%) and the second largest variance between samples PC2 (15%) (A). Venn diagrams of the genes upregulated (B) and downregulated (C) in biofilm time points (4, 12 and 24h) compared to 0h.

Figure 4: Functional annotation of differentially expressed genes reveals up-regulation of drug transporters

Gene distribution of significantly upregulated *C. auris* genes in 24 h biofilms relative to planktonic cells, grouped into biological process (BP), cellular component (CC) and metabolic function (MF) gene ontology categories (A). Log_2 fold change of up-regulated ABC and MFS drug transporters within 24 h biofilms (B). All GO terms have a *p*.value of <0.05 based upon the GOSep hypergeometric distribution test.

Figure 5: Efflux pump activity is increased in *Candida auris* biofilms

Candida auris biofilms were grown for 4, 12 and 24 h in black-bottomed 96 well plates. In addition, planktonic cells were standardised to 5×10^7 cells/mL and all cells were incubated with 100μg/mL of Ala-Nap and fluorescence measurements were read at 30

sec intervals over 60 min (Ex_{355}/Em_{460}). Data represents the mean + SD of 4 isolates repeated on 3 independent occasions. Data presented is relative fluorescence units normalised per individual cell. *$p<0.05$, **$p<0.01$, ND – not detectable

Figure 6: Formation and development of *Candida auris* biofilms

Schematic representation of the transcriptional mediators of the three main stages of *C. auris* biofilm development: adherence to yeast cells to surface (early phase), proliferation (intermediate phase) and maturation into a structured biofilm (mature phase).

Table 1. Summarised statistics for the transcriptome assembly of *Candida auris*, the alignment rate of raw reads to the transcriptome and also a summary of the Trinotate functional annotation.

All reads	**414364539**
Reads after trimming	411626529
Total assembled bases	11593681
GC content %	45.35
Total trinity "genes"	5848
Total trinity "transcripts"	5889
Contig N50 (bp)	3488
Median contig (bp)	1308
Average contig (bp)	1983
	Reads aligned
Reads aligned 1 time	393124946 (95.51%)
Reads aligned > 1	9368727(2.28%)
Overall	402493673 (97.78%)
Functional annotation	No. Transcripts
Swissprot matches BLASTx	3200
Swissprot unique Proteins BLASTx	3176
Swissprot matches BLASTp	3041
Swissprot unique proteins BLASTp	3019
TmHMM	701
SignalP	202
Gene Ontology	3085
KEGG	2889

Table 2. Assessment of *Candida auris* transcriptome assembly by benchmarking universal single-copy orthologs (BUSCO).

	Ascomycota	**Saccharomyceta**	**Saccharomycetales**
% complete	94	91.4	91.7
% complete single copy	93.4	90.5	90.9
% complete duplicated	0.6	0.9	0.8
% fragmented	3.4	4.8	4.6
% missing	2.6	3.8	3.7
Total number of genes	1315	1759	1711

Table 3. Up-regulated biofilm and resistance associated genes.

		Fold change compared to planktonic (log_2)		
Gene ID	**Function**	**4 hr**	**12 hr**	**24 hr**
IFF4	Adhesion	2.29	5.01	3.62
PGA26	Adhesion	2.02	3.90	2.55
PGA52	Adhesion	2.22	2.38	2.42
CSA1	Adhesion	3.87	6.47	6.43
PGA7	Adhesion	-	3.94	4.82
HYR3	Adhesion	-	-	2.06
ALS5	Adhesion	-	-	3.82
RDC3	Efflux pump	-	4.29	3.91
SNQ2	Efflux pump	-	2.63	3.42
CDR1	Efflux pump	-	2.30	3.19
YHD3	Efflux pump	-	2.14	2.15
MDR1	Efflux pump	-	-	2.3
KRE6	Extracellular Matrix	-	3.92	3.09
EXG	Extracellular Matrix	-	2.69	2.26
SAP5	Hydrolytic enzyme	-	-	2.19
PLB3	Hydrolytic enzyme	-	-	2.13

Table 4. Inhibition of efflux pumps increases azole susceptibility

Fluconazole ($SMIC_{50}$)						
	12 h			24 h		
Isolate number	**+ EPI***	**- EPI**	**Fold change**	**+ EPI**	**- EPI**	**Fold change**
NCPF 8971	16	64	4	16	>128	≥8
NCPF8973	2	32	16	8	64	8
NCPF 8984	16	>128	≥8	64	>128	≥2
NCPF 8990	8	32	4	16	64	4

*****EPI - efflux pump inhibitor